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The long term goals of this research are to understand the mechanisms by which *NF1* and its partners control growth using the Drosophila peripheral nerve as our assay system. This system is advantageous because we can apply a number of powerful molecular genetic methodologies that are not available in other systems. This project addresses four specific aspects of growth control, two of which were begun during these first twelve months of funding. First, we found that a reduction in PKA activity did not suppress the growth-promoting effects of Ras^{V12} as we predicted. However, in preliminary findings, we report that, as predicted, expression of *NF1* specifically within the peripheral glia can enhance the effects of Ras^{V12}, and the growth-promoting effects of a constitutively active PKA is epistatic to the growth-suppressing effects of mutations in *NF1*. Second, we found that activity of PI3 kinase, a known mediator of Ras signalling, is both necessary and sufficient to promote perineurial glial growth, and that activity of Akt, a kinase activated by PI3 kinase, is necessary for the growth-promoting effects of PI3 kinase. These results demonstrate that the PI3 kinase pathway is an essential mediator of the growth-promoting effects of Ras within peripheral nerves.

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INTRODUCTION

Over the last several years, my lab has been developing the Drosophila peripheral nerve as a system with which to identify and study the signalling pathways controlling growth of the perineurial (outer) glial layer. To accomplish this goal, we apply the various molecular genetic methodologies uniquely available in Drosophila; we hope that these methodologies will enable us ultimately to identify all of the relevant genes that interact with *NF1* to control growth, and place *NF1* and these partner genes in as complete a mechanistic context as possible. Then this mechanism could be tested and refined in systems more similar to humans but more difficult to work with (e.g. the mouse). Because all of the experiments are performed on the acutely dissected third instar larva, there are no complications or caveats associated with experimentation on cell culture systems, and we assay the entire nerve cross section as it exists within the whole organism. We thought that a more complete mechanistic understanding of growth control within peripheral nerves would greatly facilitate the ability to design drugs able to combat neurofibromas. Within this larger context, I proposed four different tasks to investigate various aspects of the genetic control of growth within peripheral nerves. These tasks involve elucidation of the relationship among Neurofibromin, pushover, and protein kinase A, as well as the identification of signalling pathways downstream of Ras that affect growth within peripheral nerves. For this funding period (months 1-12) I proposed to focus on task #1 and task #4. I proposed to initiate Tasks #2 and #3 during the second and third years of funding.

BODY

Task one: Testing the possibility that Neurofibromin activates PKA. I proposed to determine: first, if expression of $NF1^{+}$ specifically in peripheral glia suppressed the effects of $NF1^{P2}$ on perineurial glial growth, second: test if overexpression of *NF1* in peripheral glia enhanced the effects of Ras^{V12} on perineurial glial growth, third: test if loss of function mutations in *PKA* suppress the effects of Ras^{V12} on perineurial glial growth, and fourth: test the prediction that the constitutively active *PKA* called *PKA-C1** is epistatic to $NF1^{P2}$ in its interaction with Ras^{V12}. The results of these experiments are summarized in Figure 1 below.

Does expression of *NF1* specifically in the peripheral glia suppress the effects of $NF1^{P2}$ on perineurial glial growth? Expression of Ras^{V12} in peripheral glia thickens perineurial glia, and this effect is suppressed by the $NF1^{P2}$ mutation. To determine if lack of *NF1* specifically within the peripheral glia was responsible for this suppression, we introduced a *UAS-NF1* transgene into larvae carrying both *gli-GAL4* and $UAS\text{-}Ras^{V12}$, all in the presence of the $NF1^{P2}$ mutation. In these larvae, wildtype *NF1* would be expressed only in the peripheral glia; the rest of the larval cells would remain mutant for *NF1*. We found that, indeed, expression of $NF1^{+}$ in the peripheral glia rescued this mutant effect of $NF1^{P2}$. In particular, perineurial glial thickness was increased from 1.7 μm in the absence of *UAS-NF1*, to 2.6 μm in the presence of *UAS-NF1* (compare lanes 2 and 3, figure 1 below). These results suggest that loss of *NF1* specifically in the peripheral glia causes the suppression of the effects of Ras^{V12}.

Does overexpression of *NF1* specifically in the peripheral glia enhance the effects of Ras^{V12} on perineurial glial growth? To address this question, we co-expressed Ras^{V12} and *NF1* in peripheral glia, and compared perineurial glial thickness to larvae expressing Ras^{V12} alone. We found that there was no significant difference in perineurial glial thickness between the two genotypes (compare lanes #1 and #4, Figure 1 below), suggesting that our hypothesis was incorrect: overexpressing *NF1* in peripheral glia does not enhance the effects of Ras^{V12}.

Does reduction in PKA activity suppress the effects of Ras^{V12} on perinurial glial growth?

Expression of a constitutively active PKA enhances the effects of Ras^{V12}, Neurofibromin activates PKA (Tong et al., 2002), and the $NF1^{P2}$mutation suppresses the effects of Ras^{V12}. These observations led to the prediction that reduction in PKA activity would suppress the effects of Ras^{V12}. To address this question, we measured perineurial glial thickness in larvae expressing Ras^{V12} and heterozygous for a *PKA* null mutation (PKA^{H2}). This allele is expected to reduce PKA activity in the larva by 50%. We found no effect on perineurial glial growth (compare lanes #1 and #5 in Figure 1, below). Then we replaced the PKA^{H2} allele for this experiment with two alleles expected to reduce PKA activity: a deletion of *PKA* called gamma-15, which is expected to reduce PKA activity by 50%, as well as a transgene expressing a dominant-negative PKA allele called *BGO*: this transgene is a mutant form of the PKA regulatory subunit which fails to bind cAMP, and thus constitutively represses the endogenous, wildtype PKA. Again, we found no effect of this reduction in PKA activity on perineurial glial growth (compare lanes #1 and #6 in Figure 1 below).

Figure 1: Effects of altered *NF1* activity on perineurial glial growth in larvae expressing Ras^{V12}

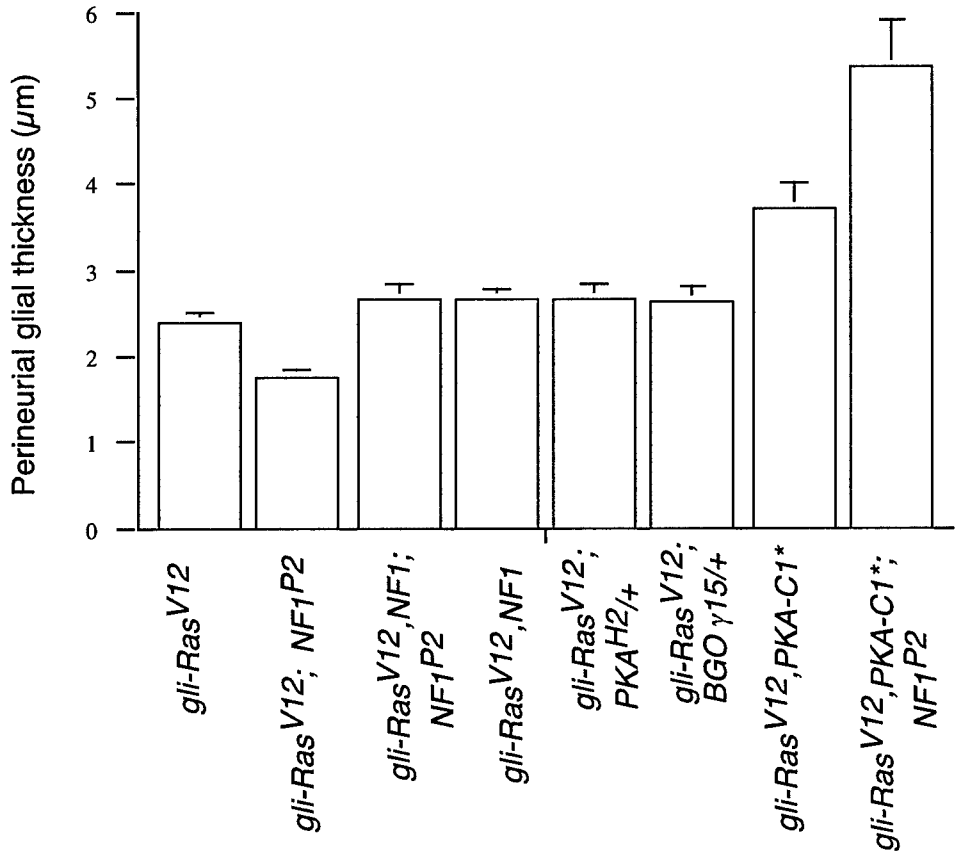

Figure 1: Mean perineurial glial thickness (μm, +/- SEM) is shown along the Y axis for the genotypes indicated along the X axis. The following pairwise combinations had statistically significant differences (Student's unpaired t-test): For *gli-*Ras^{V12}*; NF1*^{P2} (lane #2), n= 41 vs. *gli-*Ras^{V12}*-NF1; NF1*^{P2}(lane #3), n=30, $p < 0.0001$; for *gli-*Ras^{V12}*,PKA-C1*; NF1*^{P2}(lane#8), n=16 vs. *gli-*Ras^{V12}*; NF1*^{P2} (lane #2), n=41, $p < 0.0001$. For *gli-*Ras^{V12}*,PKA-C1*(lane #7) n=41 vs. *gli-*Ras^{V12}*,PKA-C1*; NF1*^{P2}(lane#8), n=16, p=0.0056.

Is expression of the constitutively active PKA epistatic to $NF1^{P2}$ in its interaction with Ras^{V12}?
$NF1^{P2}$ suppresses the effects of Ras^{V12}, whereas expression of the constitutively active PKA (called PKA-C1*) enhances the effects of RaS^{V12}. If $NF1^{P2}$ exerts its suppression by reducing [cAMP] and thus PKA activity, then expression of PKA-C1* is predicted to be epistatic to $NF1^{P2}$ because PKA-C1* does not require cAMP for activity. We tested this possibility by co-expressing Ras^{V12} and PKA-C1*in peripheral glia in an $NF1^{P2}$mutant background. We found that, as predicted, *PKA-C1** was epistatic to $NF1^{P2}$: The perineurial glia, in an $NF1^{P2}$ background and in the presence of both Ras^{V12} and PKA-C1* was extremely thick, actually even thicker than in an $NF1^{+}$ background (compare lanes#2, #7 and #8 in Figure 1).

Overall conclusions, Task one: Our observation that overexpression of *NF1* in peripheral glia has no effect on perineurial glial growth is not inconsistent with the mechanisms proposed in the grant application. This observation probably means that normally, Neurofibromin levels are not limiting for the signalling pathways in which it operates; thus, overexpression is phenotypically silent. The inability of reductions of PKA to suppress the effects of Ras^{V12} is not consistent with the central hypothesis. One possibility is that we are unable to lower PKA activity sufficiently to observe the anticipated suppression of the effects of Ras^{V12}. Unlike *NF1*, *PKA-C1* is an essential gene, so a reduction of PKA to zero kills Drosophila prior to the third instar larval stage that we assay. In this view, we are unable to lower PKA activity sufficiently to confer suppression and still retain viability. An alternative possibility, of course, is that Neurofibromin does not act through PKA.

The ability of *NF1* to rescue $NF1^{P2}$ when expressed only in the peripheral glia is consistent with our central hypothesis, as is the demonstration that *PKA-C1** is epistatic to $NF1^{P2}$, However, I have noticed that there is a lot of larva to larva variability associated with *NF1* mutations which makes me want to move cautiously in reporting these results. It is possible that some of this variability reflects genetic background effects; such effects are also observed with *NF1* mutations when the small size phenotype is assayed. We are currently isogenizing the transgenes and mutations listed in this report by back-crossing five times to our isogenic wildtype strain. Then, the stocks listed here will be re-constructed and the data re-collected. If similar results are obtained, then I will consider the data believable and will then be willing to publish.

Task four: Identification of additional Ras signalling components regulating perineurial glial growth. I proposed to conduct experiments for this task during the entire period of the award. In this task, I proposed to determine if Ras acted through the Raf-MAP kinase pathway, or the PI3 kinase, to exert its non-autonomous, growth promoting effects. After this identification was successfully completed, I then proposed to follow the identified signalling pathway further downstream by testing the effects of altering the activity of known downstream components. So far, we have demonstrated that PI3 kinase activity is both necessary and sufficient to mediate the nonautonomous, growth promoting effects of Ras. Further, we have found that the kinase downstream of PI3 kinase (called Akt or PKB) is also necessary for this growth promoting effect. The results of these studies are described in detail below.

First, to see if Ras acted through Raf to promote perineurial glial growth, we expressed a constitutively active *Raf* specifically in peripheral glia by driving *UAS-Raf^{gof}* expression with *gli-GAL4*. We found that Raf^{gof} expression had little, if any, growth promoting effect on the perineurial glia: Perineurial glial thickness was 1,94 +/- 0.1, n=20 (data not shown). This value is not very different from

values we record from various control larvae. Therefore, we decided to put Raf aside for the time being and test effects of PI3 kinase.

To determine if PI3 kinase activity was necessary to mediate the Ras growth-promoting effect, we tested to see if a reduction in PI3 kinase activity could suppress the growth-promoting effects of the constitutively active Ras^{V12}. We found that introducing the heteroallelic loss of function PI3 kinase mutations $PI3K^{A}$ (a deletion of PI3 kinase) and $PI3K^{2HI}$ (a strong hypomorph) significantly suppressed the effects of Ras^{V12} expression (Figure 2 below). This result demonstrates that PI3 kinase activity is necessary for the growth-promoting effects of Ras^{V12}. However, these data do not identify the cell type in which PI3 kinase must act: PI3 kinase could be required in the peripheral glia, the perineurial glia, or both.

To test the possibility that PI3 kinase activity is necessary in the peripheral glia, we co-expressed both $Ras^{V12}$and a dominant-negative *PI3K* mutation specifically in the peripheral glia. We found that expression of the dominant-negative *PI3K* significantly suppressed the effects of Ras^{V12} on perineurial glial growth (Figure 2 below). These results demonstrate that PI3 kinase activity is required in the peripheral glia. These results leave open the possibility that PI3 kinase activity might be required in the perineurial glia as well.

Figure 2: PI3 kinase activity is necessary for the Ras^{V12}-induced perineurial glial overgrowth

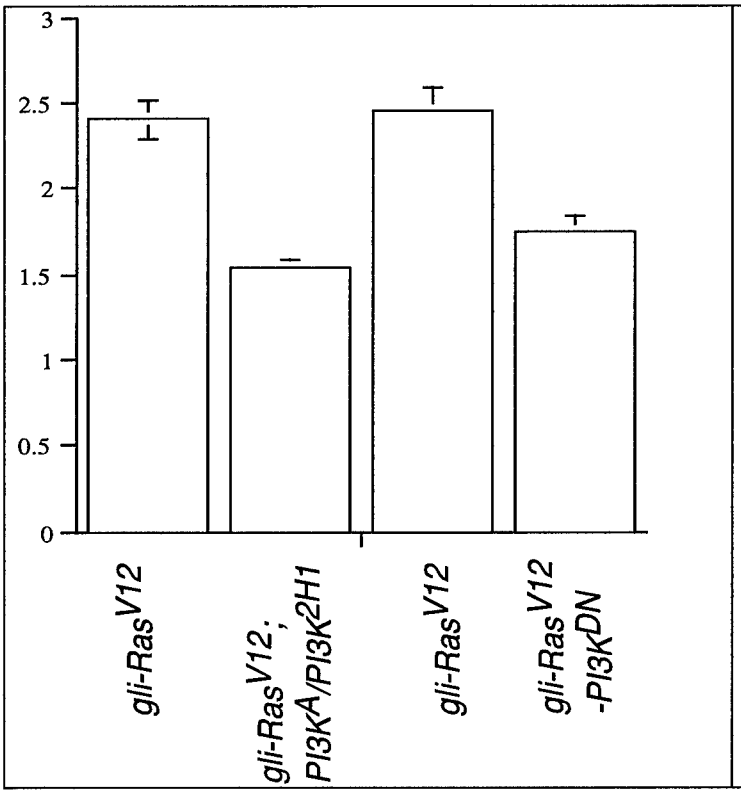

Figure 2. Perineurial glial thickness (μm) is shown along the Y axis. The X axis specifies genotypes. Means +/- SEMs are shown. The following pairwise combinations had statistically significant differences. For *gli-Ras^{V12}*, (lane #1), n=50, vs *gli-Ras^{V12}; $PI3K^{A}/PI3K^{2HI}$*, n=85, $p< 0.0001$. For *gli-Ras^{V12}* (lane #3), n=72 vs. *gli-Ras^{V12}-$PI3K^{DN}$*(n=49), $p< 0.0001$.

Figure 3: PI3 kinase activity within the peripheral glia is sufficient to promote Akt-dependent perineurial glial growth

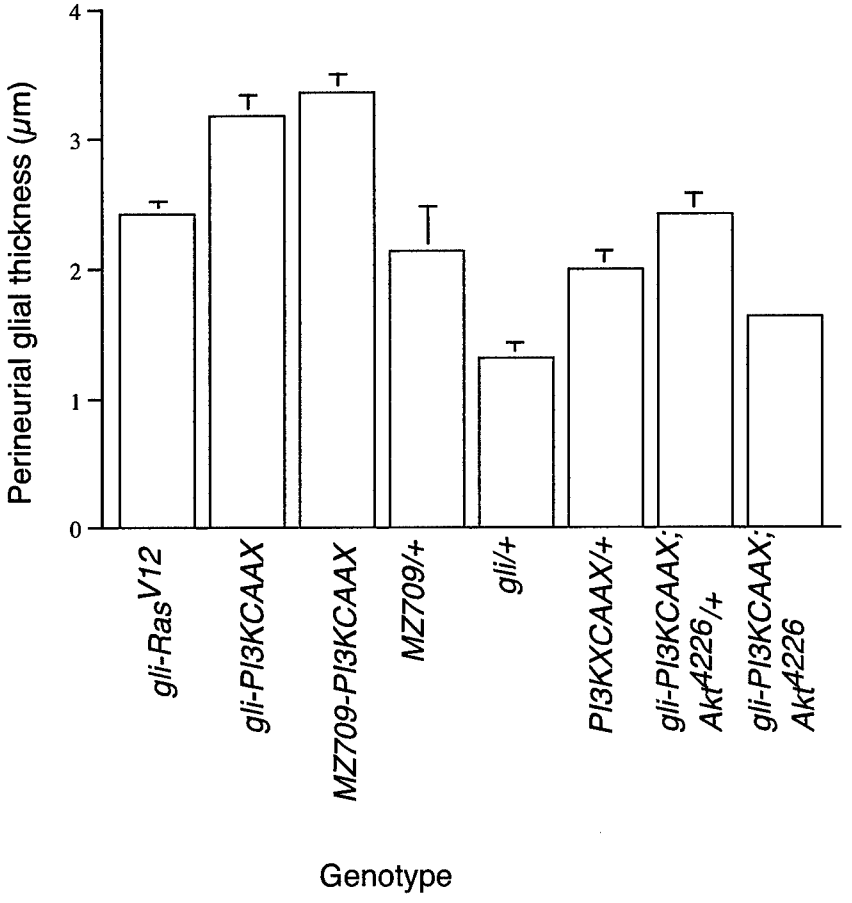

Figure 3. Perineurial glial thickness (μm) is shown along the Y axis. The X axis specifies genotypes. Means +/- SEMs are shown. The following pairwise combinations had statistically signifcant differences. *gli-PI3KCAAX* (n=42) vs. *gli*/+(n=21), p<0.0001, vs. *PI3KCAAX*/+ (n=21), p<0.0001, vs. *gli-PI3KCAAX; Akt*^{4226}(n=29), p=0.0024; and vs. *gli-PI3KCAAX; Akt*^{4226}(n=29), p<0.0001; *MZ709-PI3KCAAX* (n=27) vs. *MZ709*/+ (n=17), p=0.0034, and vs. *PI3KCAAX*/+(n=48), p<0.0001.

To determine if PI3 kinase activity was sufficient to promote perineurial glial growth nonautonomously, we expressed a constitutively active PI3 kinase called PI3KCAAX specifically in the peripheral glia. We found that expression of PI3KCAAX with either of two *GAL4* drivers that express in the peripheral glia but not the perineurial glia (*gli-GAL4* and *MZ709*) was sufficient to confer a greatly thickened perineurial glia (Figure 3 above). In contrast, expressing either *GAL4* driver in the absence of the *PI3KCAAX* transgene, or expression of the *PI3KCAAX* in the absence of the *GAL* driver, did not confer increased perineurial glial thickness. These results demonstrate that PI3 kinase activity is sufficient to increase perineurial glial growth cell nonautonomously.

PI3 kinase has several downstream effectors. One prominent effector involved in growth control is the kinase called Akt, or PKB. This kinase possesses pleckstrin homology domain, which enables its

membrane localization upon generation of PIP3, the product of PI3 kinase activity. To determine if Akt activity is necessary to mediate the growth-promoting effect of PI3 kinase, we tested the effects of *Akt* mutations on PI3 kinase-mediated perineurial glial overgrowth. We found that if we replaced one copy of Akt^{+} with a strong hypomorphic *Akt* allele (Akt^{4226}) we observed modest, but significant suppression of the PI3KCAAX-induced perineurial glial overgrowth (Figure 3, compare lanes 2 and 7). However, if we replaced both Akt^{+} alleles with Akt^{4226}, we observed an almost complete suppression of this phenotype (Figure 3, compare lanes 2 and 8). These results demonstrate that Akt is necessary to mediate the PI3KCAAX-induced perineurial glial growth phenotype. However, these results do not indicate if Akt is acting in the peripheral glia, the perineurial glia, or both.

FUTURE GOALS FOR TASK #4;

First, we will determine if Akt is required in the peripheral glia to mediate the PI3KCAAX-induced growth signal. To accomplish this goal, we have obtained two lines of flies bearing independent insertions of wildtype *Akt* under the transcriptional control of the *GAL4 UAS*. We are currently constructing the fly lines enabling the co-expression of *Akt* and *PI3K* specifically within peripheral glia, in an Akt^{4226} mutant background. If Akt is required at least in part within peripheral glia, then expressing wildtype *Akt* in peripheral glia should rescue, at least in part, the Akt^{4226}-mediated suppression of the PI3KCAAX growth-promoting effect.

We are also planning to test effects of known targets of Akt. One prominent Akt target involved in nonautonomous effects of PI3 kinase and Akt is the transcription factor FOXO. Akt phosphorylates FOXO, which inactivates FOXO by preventing entry into the nucleus. We have received fly lines from Marc Tatar (Brown University) that carry wildtype or constitutively active (can't be phosphorylated by Akt) *FOXO* transgenes under the transcriptional control of the *GAL4 UAS*. We are currently constructing the fly lines required to test the hypothesis that expression of a constitutively active FOXO will suppress the growth-promoting effects of PI3KCAAX.

KEY RESEARCH ACCOMPLISHMENTS

PI3 kinase activity within the peripheral glia is both necessary and sufficient to promote perineurial glial growth.

A kinase activated by PI3 kinase, called Akt, is necessary to mediate the effects of PI3 kinase.

REPORTABLE OUTCOMES

1. Presentation entitled "Evidence that PI3 kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves", Aspen, CO, May 23-May 25, 2004.

CONCLUSIONS

I report progress on the two tasks performed during this period of funding. On task #1, we report both negative and positive findings. The negative findings include: inability to find evidence that overexpression of *NF1* within peripheral glia could enhances the growth-promoting effects of $Ras^{V12=}$ expression, and inability to find evidence that reduction in PKA activity could suppress the growth-promoting effects of Ras^{V12} expression. The positive findings include: evidence that expression of *NF1*

specifically within the peripheral glia could reverse the suppression of the growth-promoting effects of Ras^{V12} expression, and evidence that the effects on growth of the constitutively active PKA are epistatic to the opposite effect of the *NF1* mutation. Unfortunately, however, for unknown reasons, there is a large degree of variability in glial thickness in larvae expressing Ras^{V12}, which is tending to obscure effects of introduced mutations and transgenes. The basis for most of this variability appears to come from genetic background effects. To address this difficulty, we are currently backcrossing the relevant mutations and transgenes into our isogenic wildtype fly line for retesting. For this reason, I consider these results to be preliminary until they are confirmed through testing of isogenic lines.

Our studies on task #4 has been much more successful than our studies on task #1. For unknown reasons, the effects of the PI3KCAAX transgene appear to be much less variable than effects of the Ras^{V12} transgene, so we have been able to obtain convincing and publication-quality data on experiments in task #4. So far, we have found that PI3 kinase activity within the peripheral glia is both necessary and sufficient to promote perineurial glial growth, and further, we show that the kinase activated by PI3 kinase (called Akt) is necessary to mediate this signal. In my opinion, this is the most significant finding of the first year of funding. The goal of my lab is to understand all of the molecular events occurring within peripheral nerves that control growth. In my opinion, our demonstration that PI3 kinase and Akt play major roles in this process, a possibility that was only hypothesized in the past, represents a novel and important finding, and demonstrates the validity of our approach.

These results are not meant to imply that the Raf pathway is unimportant; although in our hands, manipulation of Raf activity causes more modest effects on nerve growth than comparable manipulations of PI3 kinase, we have some preliminary data that Raf activity within peripheral glia also contributes to perineurial glial growth. For example, in data that was obtained too recently to incorporate into the main data set shown above, we have preliminary data that a dominant- negative Raf expressed in the peripheral glia can moderately, but significantly, suppress the growth-promoting effects of PI3KCAAX. We are looking forward in the next three years to continuing our analysis of the effects of manipulating these signalling pathways.

REFERENCES

Tong, J., Hannan , F., Zhu, Y., Bernards, A., and Zhong, Y. 2002. Neurofibromin regulates G protein–stimulated adenylyl cyclase activity. Nat. Neurosci. **5:** 95-96.

APPENDIX

1) Abstract of presentation to the NNFF Consortium on NF1 and NF2, entitled " Evidence that PI3 Kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves" (Aspen, CO, May, 2004).

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ABSTRACT FORM

TOPIC: Signaling pathways in NF and TSC

TITLE: Evidence that PI3 Kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves

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Drosophila peripheral nerves comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). We have been using these nerves as an assay platform to test the effects of mutations and transgenes on perineurial glial growth. It was previously shown that perineurial glial growth in third instar larval nerves is regulated by a number of genes including *push*, which encodes a large Zn^{2+}-finger-containing protein, *amn*, which encodes a putative neuropeptide related to PACAP, and *NF1*. We found that expression of the constitutively active Ras^{V12} transgene specifically in peripheral glia increased growth within the perineurial glia. This result demonstrates that Ras activity is sufficient to promote perineurial glial growth, and that Ras can act cell nonautonomously. Surprisingly, we found that the $NF1^{P2}$ null mutation suppresses these effects of Ras^{V12}, suggesting that *NF1* has a relevant activity that promotes, rather than inhibits, perineurial glial growth. The possibility that activation of adenylate cyclase represents this second activity is supported by the observation that expression within peripheral glia of any of three genes expected to increase protein kinase A (PKA) activity (a constitutively active PKA, the *amn*-encoded PACAP-like neuropeptide, or a constitutively active $G_{\alpha}s$) strongly enhances the growth promoting effects elicited by Ras^{V12} alone. These results are consistent with the possibility that a signalling pathway from the Amn neuropeptide through $G_{\alpha}s$, Neurofibromin, and PKA strongly potentiates the effectiveness of constitutive Ras activity on perineurial glial growth.

To identify the downstream components that mediate the effects of Ras, we tested the effects of constitutively active *Raf* and *PI3 Kinase* transgenes on perineurial glial growth. We found that expression of a constitutively active *PI3 Kinase,* but not a constitutively active *Raf*, strongly increased perineurial glial growth, suggesting the possibility that PI3 Kinase is an important mediator of the growth-promoting effects of Ras in peripheral nerves.